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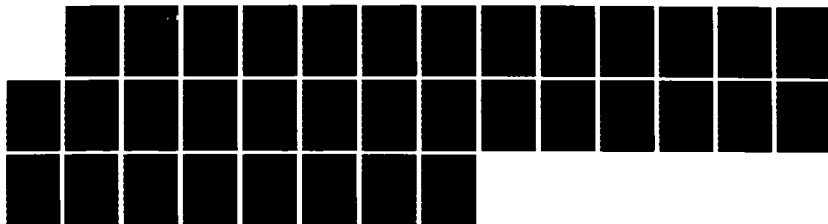
DIRECT INTERACTION BETWEEN AUTONOMIC NERVES AND THE  
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AND DENTISTRY D L FELTEN 05 AUG 86 N00014-84-K-0488

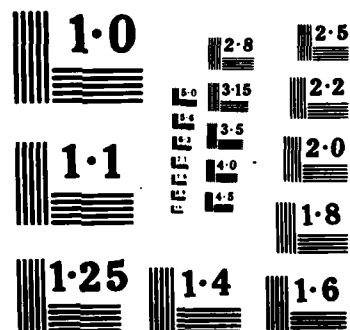
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19. ABSTRACT (Continue on reverse if necessary and identify by block number) This project examines interactions between autonomic nerves and the immune system. Noradrenergic sympathetic nerves are present in lymphoid organs, particularly in T cell compartments and macrophage zones of spleen and lymph nodes. Neurochemical studies reveal both the presence and availability of norepinephrine in spleen and lymph nodes, and the presence of acetylcholine in spleen. Following surgical ablation or neurochemical denervation of the noradrenergic nerves innervating spleen and lymph nodes, many immune parameters are altered, including primary and secondary antibody responses, mitogen responses, delayed-type hypersensitivity reaction, and additional in vitro measurements. We have begun studies with cold exposure in mice, where this aversive condition can lead to suppressed immune responses. Our present findings indicate that the autonomic nerves innervating spleen and lymph nodes are necessary for immunocompetence, and that neurotransmitters used by these nerves may be a class of molecules that exert an immunomodulatory influence in lymphoid organs.						
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## INTRODUCTION

This project examines interactions between the autonomic nervous system and the immune system in young adult male C3H and BALB/c mice. Evidence from our laboratory and others has revealed direct autonomic innervation of the parenchyma of lymphoid organs by noradrenergic and putative cholinergic nerves, with a particular concentration in regions where T lymphocytes are found. We have demonstrated changes in antibody and cell-mediated immune responses following alterations of the noradrenergic nerve supply to the spleen and lymph nodes. We hypothesize that the autonomic nerves supplying lymphoid organs represent an important mechanism through which aversive environmental conditions may lead to altered immune function. In this project, autonomic nerves and their transmitters in spleen and lymph nodes are manipulated experimentally by: (1) surgical denervation, based upon anatomical data established in our laboratory; (2) pharmacologic manipulation designed to enhance or reduce sympathetic noradrenergic innervation and parasympathetic cholinergic innervation; and (3) exposure to cold (4°), known to enhance peripheral sympathetic turnover of norepinephrine. Following these experimental manipulations, we are studying both neural and immunologic parameters. For investigation of autonomic innervation, we are utilizing light and electron microscopic methods to analyze the relationship of noradrenergic and cholinergic terminals to specific cell types in compartments of spleen and lymph nodes, and neurochemical analysis of the presence and availability of the transmitter in these compartments. For investigation of the immune responses to experimental manipulation, we are examining: (1) cell-mediated immune reactivity to contact sensitizing agents and to cell surface antigens; (2) natural killer (NK) cell function; and (3) natural resistance and acquired immunity to herpes simplex virus type 1 (HSV1). This combined effort seeks to elucidate the means by which the autonomic nervous system and the immune system interact physiologically and under aversive environmental conditions such as prolonged cold exposure.

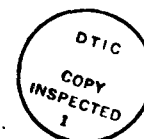
### 1. INNERVATION OF LYMPHOID TISSUE

#### a. Introduction

Recent studies from our laboratory have provided evidence for both vascular and parenchymal innervation of lymphoid tissue in murine thymus, spleen, and lymph nodes, as well as other lymphoid tissue in other species. This innervation is found in specific compartments of these lymphoid organs, and is associated with specific cell types of the immune system.

#### b. Anatomical and Neurochemical Studies of Murine Spleen

Fluorescence histochemical observations of noradrenergic nerve fibers and varicosities in lymphoid tissue of C3H, BALB/c, and other strains of mice have demonstrated the abundant presence of noradrenergic innervation of the spleen. Noradrenergic fibers entered the spleen with dense plexuses surrounding the splenic artery and its branches, and either traveled directly into the spleen with these branches, or traveled in a subcapsular plexus, and then followed trabeculae to sites where white pulp was found. In the trabeculae, only a small portion of the fibers traveled with blood vessels, as noted with combined gel-ink vascular/histofluorescence preparations. From these plexuses, noradrenergic fibers richly supplied the central arteries and their branches in the white pulp, where a majority of the splenic innervation was found. The combined vascular/histofluorescence preparations revealed some of these fibers traveling with the central arterial system, and other fibers extending beyond the vasculature into parenchymal regions of white pulp, or alongside capillary networks in the zones of T lymphocytes. Some plexuses extended outward to the penicillar arterial system at the marginal zones and ended at the boundary with the red pulp.



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Combined immunohistochemical localization of tyrosine hydroxylase (as a marker for the noradrenergic nerves) and markers for cells of the immune system (T cell markers, B cell markers, macrophage markers) revealed the presence of the noradrenergic fibers in several specific compartments of the spleen, more obviously discernable with immunohistochemical methods than with catecholamine fluorescence histochemistry. The tyrosine hydroxylase (TH)-positive fibers were found within the periaarteriolar lymphatic sheath (PALS), at the inner region of the marginal zone, and in the perifollicular zone. The TH-positive fibers were found in direct association with T cells (both T helper and T suppressor cells) and with macrophages, and only occasionally ventured into the follicular territory of the B cells. Although no striking differences were noted in the distribution and appearance of noradrenergic varicosities along the vasculature or within the parenchyma in the spleens of adult C3H, BALB/c, and other strains of mice, there were marked age-related changes in innervation. The spleen and lymph nodes of both mice and rats showed an age-related decline in noradrenergic innervation, with more than 50% depletion of the norepinephrine content and even greater depletion in the number of varicosities in the white pulp. In striking contrast to the spleen and lymph nodes, the thymus demonstrated an increased density of noradrenergic fibers with aging. We were able to mimic this situation of increased density of innervation using treatment of C3H mice with immunosuppressive drugs, including cyclophosphamide and hydrocortisone. With these drugs, both the thymus and spleen demonstrated a considerable loss of lymphocytes, but the noradrenergic fibers remained intact, suggesting that the nerves had merely accommodated to the new geometry of the depleted lymphoid organs. We do not have an explanation at present why the spleen and lymph nodes show an age-related decline in innervation while the thymus shows an increased density of innervation. In an additional mouse model, the NZB autoimmune strain, we also found a diminished noradrenergic innervation, evident quite early in life, at 8 months of age. There also were some age-related differences in thymic innervation and lymph node innervation, but the splenic innervation in adults appeared to be stable.

The noradrenergic terminals appeared to end blindly among fields of lymphocytes, particularly in perivascular zones where T lymphocytes have been identified with immunohistochemical methods for the Thy-1 marker. Preliminary electron microscopic observations of these terminals have shown the presence of dense-core vesicles, but no presynaptic specializations. These varicosities ended adjacent to lymphocytes, but rarely showed any classical synaptic specializations. We do not consider such specializations a necessity for potential transmitter-lymphocyte interactions, in view of the mobile nature of this unique target tissue, and in view of our neurochemical findings that suggest high levels of norepinephrine available throughout the white pulp as a paracrine substance. Such non-specialized noradrenergic endings are comparable to those found in the liver adjacent to hepatocytes; norepinephrine from these terminals can exert a marked functional influence on glycogenolysis, hyperglycemia, and subsequent gluconeogenesis despite the absence of synaptic specializations.

At present, we are undertaking light microscopic studies of the relationships of noradrenergic varicosities to T lymphocytes and their subsets, macrophages, and NK cells, using immunohistochemical techniques for the localization of specific surface markers. These studies will be continued and extended to examine the effects of immunization or pharmacological and surgical manipulation of the innervation, to better understand the conditions under which these specific cell types associate with nerve terminals in the splenic white pulp.

We have evaluated quantitatively the compartmentation and distribution of noradrenergic varicosities in the white pulp with fluorescence histochemistry and computerized morphometric techniques, and have achieved a combined histochemical/neurochemical analysis with a new adjacent section method. We can map the distribution of varicosities morphometrically in a single region of splenic white pulp in a single cryostat section, followed by immunohistochemical analysis of cell surface markers

for cell subsets of the immune system. In the next adjacent cryostat section, we use a fine needle, flattened and hollowed to micropunch a small disc of frozen tissue from that same region of white pulp, and measure the norepinephrine content in that single disc with high performance liquid chromatography with electrochemical detection (LCEC). This gives us the opportunity to compare morphological and neurochemical data in adjacent sections within a single region with defined architecture and defined cellular populations. We currently are evaluating the distances, dimensions, and distributions of these nerve-lymphocyte relationships at the light microscopic level.

We are undertaking electron microscopic observations with a new Zeiss EM902 Electron Energy Loss Spectroscopy (EELS) System with IBAS Image Analyzer, obtained through the DOD URIP Program. This system provides a new technology which is available only in a few laboratories at present. We are able to view directly low atomic-weight elements on the basis of their electron energies and their subsequent response to the electron beam. In preliminary observations of fluorinated compounds, we are able to view these substances directly at the ultrastructural level strictly on the basis of the spectra from the EELS measurements. This technology opens up the opportunity to localize 2 or more compounds (eg. enzymes, surface antigens) in a single thin section at the ultrastructural level. We also have obtained a new Reichert ultramicrotome for this project, permitting us to cut the requisite thin sections of 30 nm, one of the critical limitations of this technique. Fortunately, results with these thin sections have been excellent, and our sections are suitable for observations. Since we are breaking new ground with this technology as applied to analysis of nerve-lymphocyte relationships, we are progressing methodically, first with a single compound and a single antibody, and then with multiple localizations. We will use boronated antibodies and halogenated ligands and markers where possible, since they are readily viewed with the EELS system, and are elements that are not normally present in biological tissue in appreciable amounts. We will continue to extend our light microscopic findings to this ultrastructural level. Our ultimate goal is to understand the relationship between the noradrenergic nerve terminals and the various cells of the immune system, in specific sites in the spleen and lymph nodes, and to understand the conditions under which these associations may be altered. From our preliminary observations in neonatal and developing mice, it is clear that the association of noradrenergic nerves and cells in lymphoid organs appears after birth, and can change with age and with altered conditions of both the nervous system and the immune system.

Neurochemical studies continue to be an integral part of our analysis of innervation of lymphoid tissue, and have been refined considerably in the past year. LCEC analysis reveals levels of  $0.39 \pm 0.04$  (mean  $\pm$  SEM) ng of NE/ mg of spleen. We have demonstrated that the NE is confined almost entirely to the neural compartment of the spleen, as shown by denervation and pharmacologic studies. Surgical ablation of the coeliac/superior mesenteric plexus results in total depletion of NE from the spleen and the disappearance of fluorescent profiles. We found these ganglia to be the site of cell bodies supplying postganglionic noradrenergic fibers to the spleen, using retrograde tracing methods with fluorogold. Chemical denervation with 6-hydroxydopamine (6-OHDA) reduced splenic NE content to approximately 5% of control levels. Therefore, effects of denervation in subsequent studies are mainly due to changes in neural NE. We have refined our assay capabilities, as described above, to include the measurement of NE in a single micropunched disc of white pulp from a single cryostat section, allowing direct comparison with morphometric evaluation of varicosity numbers and distribution. These measurements will continue to be an important objective in the renewal period.

Dr. Suzanne Felten has undertaken preliminary studies of the availability of NE in the spleen of rats using a modification of in vivo dialysis methods. Using a fine piece of dialysis tubing looped through a thin needle, connected to a Harvard infusion pump (set a 1-2 microliters/minute of flow), measurements of released NE are possible. The rat was selected for preliminary studies because of its larger size than the mouse. The resultant measurements demonstrate ongoing availability of NE from the rodent spleen. In

additional LCEC studies, we have obtained direct measurements of acetylcholine, the presumed parasympathetic neurotransmitter, in spleen. This provides the first direct evidence for cholinergic innervation of lymphoid organs. Studies using immunohistochemical methods for the localization of choline acetyltransferase, the synthetic enzyme for acetylcholine, have been unsuccessful in our laboratory and in several other laboratories where this approach was attempted. The use of acetylcholinesterase is notoriously unreliable for the localization of cholinergic fibers, and in studies of mouse spleen and rabbit appendix, we have some evidence that both acetylcholinesterase and norepinephrine may be co-localized in the same nerve fibers.

#### c. Anatomical and Neurochemical Studies of Murine Lymph Nodes (LN)

We have identified extensive noradrenergic innervation of cervical, mesenteric, and popliteal lymph nodes in several strains of mice. The noradrenergic fibers entered the nodes along vascular plexuses in the hilar region and traversed the medullary cords, where they are found adjacent to both lymphocytes and macrophages. NE varicosities then branched abundantly into both cortical and paracortical regions of the lymph nodes surrounding the germinal centers. A large subcapsular plexus of NE varicosities contributes to this innervation, particularly of cortical regions. Our use of the combined gel-ink vascular/histofluorescence preparation revealed a surprising difference between NE associations in the lymph nodes compared with the spleen. In the lymph nodes, the NE fibers are almost entirely free of vascular association in the cortical and paracortical zones where T lymphocytes reside, despite their appearance as cylindrical, tortuous plexuses of fibers. In contrast, splenic NE fibers follow the central arterial network into the white pulp before branching into the parenchyma. In lymph node T cell zones, frequent fine linear profiles are found in the parenchyma. We are carrying out computerized morphometric analysis of the density and distribution of the NE terminals in association with T lymphocyte subsets, B lymphocytes, and macrophages. We will extend these studies to the ultrastructural level using the Zeiss EM902 EELS system.

Neurochemical analysis of NE in lymph nodes has detected concentrations in the range of 2.5-5.0 ng NE/mg protein. NE levels appear higher in mesenteric lymph nodes than in popliteal or cervical lymph nodes. In addition, strain variability exists, with levels in C57BL/6 mice higher than in C3H mice. We continue to measure these levels in functional studies, particularly in view of the marked fluctuation in the size of lymph nodes following immunization. As a particular LN compartment expands in volume, the effective NE concentration may decrease temporarily. Thus, concurrent chemical and anatomical data thus become necessary for interpretation of functional findings.

#### d. Cholinergic and Peptidergic Innervation of Lymphoid Tissue

As initially proposed, we carried out examination of spleen and lymph nodes using immunohistochemical staining of the synthetic enzyme CAT (choline acetyltransferase), an excellent marker for a cholinergic cell or fiber. We were able to obtain CAT antibodies from several sources, but none of them yielded useful information about cholinergic innervation of spleen or lymph nodes, despite their utility for central cholinergic neurons. We conclude that the CAT is either bound or protected in the peripheral nerves in lymphoid tissue, or is structurally non cross-reactive enough with the antibodies. We therefore have turned to use of specific acetylcholinesterase (AChE) staining as a marker for putative cholinergic fibers and terminals, although we are well aware of the literature, some of it from our own laboratory, suggesting that noradrenergic or other chemically specific nerve fibers may contain co-localized AChE. More promising is the direct LCEC measurement of acetylcholine.

Our studies with specific AChE staining have revealed a plexus of neural-like profiles at the thymic cortico-medullary junction, in agreement with the reports from Karen Bulloch's laboratory. We also have found abundant neural-like AChE-positive profiles in the splenic white pulp and the cortical and paracortical regions of lymph nodes. We have initiated surgical ablation studies, with various ganglionectomies and cuts of the subdiaphragmatic

vagus nerves, followed by histochemical staining for both NE and AChE in alternate sections, a combined technique developed in our laboratory during the past year of ONR support.

We will combine chemical measurement of acetylcholine with surgical ablation studies to identify the origin of cholinergic innervation to the spleen and selected lymph nodes. Of course, we still consider the possibility that parasympathetic cholinergic fibers do not innervate secondary lymphoid organs. Since the existence of ACh receptors on lymphocytes, and altered immune responses following cholinergic manipulation suggest the contrary, we will continue our proposed studies.

We also have found peptidergic innervation of thymus, spleen, and lymph nodes in the rodent, although we have not followed up our immunohistochemical work with functional studies. These peptides may be co-localized with NE or ACh, and thus may function in concert with them. While the identification of peptides such as met-enkephalin, CCK, and somatostatin in lymphoid tissue opens additional possibilities for neural modulation of immune responses, we feel that it is important for us to retain our focus on the established and classical noradrenergic and cholinergic systems.

#### e. Summary of Anatomical and Neurochemical Studies

We have examined noradrenergic innervation of murine spleen and lymph nodes with computerized morphometric techniques and have substantiated the presence of terminals in specific sites where T lymphocytes and macrophages are found. The development of a gel-ink vascular/histofluorescence method has revealed an association of noradrenergic fiber plexuses with the central arterial system of the splenic white pulp, from which varicosities branch into the parenchyma, and a surprising absence of association of noradrenergic fibers with the vasculature in paracortical and cortical regions of lymph nodes. The refinement of micropunch LCEC neurochemical analysis has permitted measurement of norepinephrine in single lymph nodes and in single micropunched discs of white pulp, adding quantitative substantiation to our anatomical data. Anatomical tracing studies have revealed the coeliac/superior mesenteric ganglionic system as the source of both the noradrenergic fibers and acetylcholinesterase-positive profiles in the spleen, thus permitting surgical denervation in addition to pharmacological manipulation of the transmitters. With combined use of fluorescence histochemistry for norepinephrine localization and immunohistochemical localization for surface markers (eg. Thy-1, Lyt-1, Lyt-2) on specific subsets of lymphocytes or other cells of the immune system, we have begun mapping the relationship between the nerve terminals and these cells. The recent installation of the Zeiss EM902 has permitted us to extend these studies to the ultrastructural level.

We also have identified AChE-positive fibers in the spleen and lymph nodes, and have undertaken studies to identify the source of these fibers, to permit surgical approaches to the parasympathetic innervation in addition to pharmacologic manipulation. We have utilized these basic data to undertake functional studies linking neural alterations with immune alterations following pharmacological or surgical manipulation, and following cold exposure.

## 2. IMMUNOLOGIC STUDIES

### a. Delayed Hypersensitivity (DH) Responses

#### (1) Contact Sensitivity to Haptens

Studies of contact sensitivity reactions to epicutaneously applied haptenic molecules were initiated during the past funding period. Early experiments were directed towards establishing the optimal conditions of the response in the mouse strains of interest, as well as confirming the immunological specificity of the reaction in our hands.



Table 1 summarizes several experiments in BALB/c mice, in which the specificity of the ear swelling response to challenge with optimal concentrations of 3 non-cross-reactive antigens was tested. Mice were painted on their shaved abdominal skin with either the trinitrophenyl (TNP), the dinitrophenyl (DNP), or the oxazolone (OXA) hapten in a chemically reactive form. An ethanol solution of picryl chloride (7%) (TNP) or oxazolone (3%) was applied once in a volume of 0.1 ml to shaved abdominal skin on day 0. Dinitrofluorobenzene (DNFB) at a concentration of 0.5% (in a 4:1 acetone:olive oil mixture) was applied twice, 0.05 ml on days 0 and 1. Four to six days later, baseline ear thickness was measured with a dial thickness gauge, and mice were challenged by painting both ears with 0.02 ml of hapten solution (1% picryl chloride or 2% oxazolone in olive oil, or 0.1 % DNFB in acetone:olive oil). Ear swelling was measured 24 hr later, and in some animals, 48 hr later as well. The response was expressed as "net ear swelling" (in inches  $\times 10^{-4}$ ), calculated as the mean of the swelling of both ears relative to day 0. The antigen-specific response was invariably greater at 24 hr, and this time was chosen as the standard for most experiments. However, in order to observe possible effects of our treatments on the rate of ear swelling or its natural decline, initial experiments using any novel manipulation will include both 24 and 48 hr measurements.

Clearly, the responses to TNP, DNP, and OXA were immunospecific in BALB/c mice, with the TNP and OXA responses being of greatest magnitude (Table 1). For this reason, and due to other considerations, including ease of hapten modification of cells in vitro, further studies were directed to TNP-specific responses. C3H mice also demonstrated potent and specific responses to TNP.

The effect of adult sympathectomy by systemic treatment with 6-OHDA on this type of DH response was tested in both C3H and BALB/c mouse strains, which we have found to be among the most sensitive to the immunosuppressive effects of denervation. In initial experiments using BALB/c mice, 3 doses of 6-OHDA were employed. Each was administered twice, 1 and 3 days prior to sensitization with TNP. Animals then were sensitized epicutaneously on day 0, and were subsequently challenged on day 5. Ear swelling was determined both at 24 and 48 hr. Two injections of 150 or 300 mg/kg 6-OHDA caused a significant inhibition of the DH response, while 100 mg/kg was without effect (Table 2). The ear swelling was reduced to a similar degree both at 24 and 48 hr.

Administration of 6-OHDA (150 mg/kg) at different time points during development of the DH response was examined in C3H mice in the next set of experiments. Treatment on days 1 and 3 before sensitization reduced the 24 hr ear swelling response by almost 50%. Unexpectedly, a single injection of 6-OHDA on day -1 inhibited the 48 hr but not the 24 hr reaction to challenge on day 5. This finding, which we cannot yet explain, requires confirmation. Such a result would be most easily attributable to an effect of denervation on the efferent rather than the afferent stage of the response. Therefore, experiments were undertaken to test the effect of 6-OHDA given after sensitization but before challenge. Animals were injected with 150 mg/kg either on day 4 or on days 2 or 4 after sensitization, followed by challenge on day 5 and measurement on days 6 and 7. Both of these treatments reduced the ear swelling response at 24 hr (Table 2). The difference was no longer apparent at 48 hr, at which time the magnitude of the response was already waning. These findings clearly imply that some aspect of the efferent phase of the DH response is dependent upon intact sympathetic innervation. A larger range of time course studies is required to determine whether the denervation prior to sensitization is acting on the afferent phase, or residually on the efferent phase as well.

These experiments do not yet allow the identification of the mechanisms which are likely to be affected by noradrenergic sympathetic nerves. Since the efferent phase is affected in the studies described above, either migration of sensitized T cells (T<sub>dh</sub>) to the challenge site, release of lymphokines by these cells, or accumulation of infiltrating mononuclear cells in response to these signals is disrupted. These possibilities will be analyzed by the use of adoptive transfer studies, as well as by testing the ability of

sensitized T cells to release lymphokines in vitro in response to antigenic stimulation (see below).

#### (2) DH to Alloantigens: Direct Response and Local Transfer

Prior to initiation of proposed studies with Herpes Simplex Virus 1, we decided to examine DH responses to major histocompatibility complex alloantigens, as highly immunogenic "foreign cell surface antigens" which generate responses analogous to those induced by viral cell surface antigens.

C3H and BALB/c mice both showed marked footpad swelling responses following subcutaneous (s.c.) immunization with allogeneic cells on day 0, and s.c. challenge into one footpad 5-7 days later (Table 3). These responses demonstrated allospecificity, with H-2<sup>k</sup>-immune BALB/c mice responding to both C3H and AKR alloantigens. Response was greatest to the immunizing cell type (C3H), suggesting that reaction to other surface antigens besides MHC probably is involved.

Table 4 shows results of local adoptive transfer experiments. Here, immune lymph node (LN) cells from mice immunized s.c. 4-5 days earlier with allogeneic spleen cells, were transferred s.c. along with the specific (or 3rd party) allogeneic cells into normal adoptive recipients. The DH response was manifest in 24 hr by swelling of the injected footpad. This response was proportional to the number of transferred Tdh cells, and was dependent on the transfer of specific immune cells. The ease of achieving local transfer of DH to alloantigens makes this an attractive candidate for examination of sympathetic neural regulation of DH. Depending upon our ability to achieve local transfer of DH to haptens in the contact sensitization model, we may employ this model to answer certain questions. The capacity to perform adoptive transfer with the cells of individual animals (rather than pooled cells) offers an important advantage, justifying the use of this additional paradigm.

#### b. Measurement of IL2 Production by T Lymphocytes

We already have initiated the study of interleukin 2 (IL2) production in vitro by TNP-sensitized lymph node (LN) cells, as well as by non-immune T lymphocytes, following stimulation by syngeneic irradiated TNP-modified spleen cells as the antigenic stimulus. In these experiments, normal or immune BALB/c lymphocytes were cultured for varying times with a standard number of irradiated, TNP-modified BALB/c spleen cells as stimulators. Supernatant fluids from these cultures were collected and stored frozen at -20° c. We established in our laboratory the colorimetric assay for IL2 as originally described by Mosmann, utilizing the IL2-dependent cell line CTLL2. The culture supernatant of rat spleen cells stimulated with Concanavalin A served as our experimental standard for determination of a maximal IL2 response. Preliminary experiments (Fig. 1) indicated that optimal IL2 production by TNP-immune LN cells, when stimulated by TNP-modified syngeneic spleen cells, occurred on day 2, with the cell concentrations used here. Supraoptimal effects, apparently due to increased consumption of the secreted IL2, were observed on day 3 with immune LN cells. Non-immune LN cells showed lower responses with higher background (unstimulated) IL2 production. Experiments are currently underway to establish the optimal conditions for study of IL2 production in response to hapten-modified self, by both normal lymphocytes and cells from contact sensitized mice.

#### c. Studies of Natural Killer (NK) Cell Function

The activity of NK cells, (which function in natural resistance to HSV1 infections), is routinely measured in vitro by a <sup>51</sup>Cr-release cytotoxicity assay on YAC-1 lymphoma target cells. We have initiated NK cell studies during this grant period. Following extensive parametric studies, we have settled on the lytic unit method using the exponential fit equation (and the "EXPFIT" computer program) to analyze our data. Effector cells are prepared routinely from the spleen and plated at 4 or more effector:target cell ratios with 10<sup>4</sup> isotope-labelled YAC-1 target cells, and lysis determined 4-6 hr later.

We have thus far examined the effects of two types of pharmacologic treatments on NK cell function. In addition to baseline activity, we also have tested poly I:C boosted NK cell activity (which results from interferon induction by poly I:C). The effect of sympathetic denervation on splenic NK activity was tested first (Fig. 2). In contrast to most of the other observed effects of 6-OHDA administered acutely to adult mice, we found that NK activity, both baseline and poly I:C-augmented, was significantly enhanced by about 2-fold, following 2 injections of 100 mg/kg 6-OHDA given 1 and 4 days before assay. When the time course of response to a single dose was monitored, we found that, while there was no change at 2 days, there was a significant enhancement of splenic NK cell activity 4 days after treatment. This effect was no longer discernable by 7 days. Further studies are necessary to determine whether: (1) these effects also are manifest on NK cells at other sites, including those not residing in innervated lymphoid organs (eg. peripheral blood, peritoneum); and (2) the effect is due to greater lytic activity per cell or an increased frequency of lytic cells in the spleen. Of major importance is the role of sympathetic innervation in the in vivo defense reactions presumably mediated by NK cells. In conjunction with another project, we currently are studying the retention or rejection of intravenously (i.v.) injected YAC tumor cells in the lung, as well as the metastasis to the lung of i.v. inoculated metastatic melanoma cells. NK cells are believed to play an important role in the early phases of these processes. As we expand our studies to the HSV1 model, we will be in a position to test the importance of interactions between sympathetic nerves, NE, and NK cell function in resistance to viral infection.

Our initial examination of the effects of chronic propranolol administration on NK cell function involved the implantation of propranolol-containing pellets s.c. into mice. Only one dose, releasing about 3 mg/kg per day, has been studied, in comparison with placebo control pellets. C3H mice were implanted with pellets and were sacrificed at approximately weekly intervals. Spleens and LN from these mice were taken for study of several lymphocyte functions (see below) as well as for NK activity in vitro. Fig. 3 shows that after 3 weeks of chronic propranolol, a significant fall in NK activity was observed. At 4 weeks, the variability of responses resulted in a lower mean value which did not reach statistical significance. It will be important to examine several doses of drug over this interval as a dose-response study. These pellets discharge their entire contents of drug by the 4th week. Therefore, we will need to monitor the return to normality of NK activity following the 4th week. It is not clear whether the reduction in NK by the 3rd week was due to a direct effect, at the level of the individual NK cell, or on population dynamics in the spleen. Our anatomical studies will aid in answering this question. The fact that T and B lymphocyte proliferative responses are not altered in the spleen at this time (see Fig. 4), suggests that the effect is restricted to certain cells. If NK cells do indeed have beta-adrenergic receptors, the blockade of which depresses cytolytic activity, then enhancement of NK activity by removal of normal noradrenergic input by 6-OHDA treatment should occur, which is what we found. These findings also would be in keeping with observations in the literature that elevation of intracellular cyclic AMP levels, which occurs following beta receptor stimulation, inhibits the lytic function of cytotoxic T lymphocytes (CTL) induced either in vitro or in vivo. Direct treatment of NK cells with adrenergic agonists or antagonists in vitro will allow us to approach some of these questions. We already have begun addressing related questions by a study of the modulatory effects of both naturally occurring and synthetic adrenergic compounds on the generation in vitro of CTL against alloantigens, as a preliminary approach to our planned study of HSV-specific CTL.

#### d. Additional Studies of T and B Lymphocyte Responses

##### (1) Lymphocyte proliferation

In conjunction with the measurement of NK cell activity, the T and B cell proliferative responses to mitogens were examined in spleens and LN of mice treated with propranolol pellets. The results appear in Fig. 4. In the spleen, no changes in the in vitro proliferative

responses were observed, with both Con A and PHA as T cell mitogens, and *Salmonella typhimurium* mitogen (STM) as a B cell stimulant. This is in clear distinction to the drop in NK cell activity in these same spleens after 3 weeks. Interestingly, in LN cells, the B cell proliferative response was significantly enhanced at 1 week, and returned to normal levels by 2 weeks, while T cell responses were unaffected.

### (2) Antibody response

Preliminary experiments (Table 5) were performed examining the effect of 3 or 4 weeks of chronic propranolol treatment on the antibody responses in spleens and LN to sheep RBC (the only antigen to which our laboratory currently has routine plaque assays). Animals were immunized either i.p. or s.c. on days 17 or 24, and the PFC response in spleen or LN was measured 4 days later, on days 21 or 28. A significant increase in the PFC response was noted in the spleen at 3 weeks and the LN at 4 weeks. Further studies are required to examine earlier times as well as to confirm whether this one week difference between spleen and LN is a consistent observation.

In preparation for the HSV1 studies, we have begun to develop an enzyme-linked immunoassay (ELISA) to measure serum antibodies to a soluble protein antigen, keyhole limpet hemocyanin (KLH). Results of the first experiment measuring IgM antibodies to KLH in control and 6-OHDA treated animals injected with 3 doses of antigen i.p. and assayed on day 5, showed differences which did not reach statistical significance due to variability between animals. These animals have since been given a secondary immunization at 3 months, and have been bled at several time points. Their sera await analysis of IgM and IgG antibodies to KLH.

### (3) Cytotoxic T lymphocyte (CTL) responses

The effect of sympathetic denervation on the development of CTL in draining LN of mice immunized s.c. in their footpads with alloantigens was examined in C3H mice. Mice received 100 mg/kg 6-OHDA on days -3 and -1, were immunized with BALB/c spleen cells on day 0, and the popliteal LN were harvested on day 5 and tested for CTL activity on  $^{51}\text{Cr}$ -labelled P815 tumor target cells. As with NK cell lysis, the % Lysis at varying effector:target ratios were fit to an exponential fit equation, and the Lytic Unit<sub>30</sub> (LU) value derived. Cytotoxic activity, expressed as both LU per  $10^6$  cells and LU per LN appears in Fig. 5. A significant reduction in CTL activity was observed. Since the cellularity of the LN was reduced by sympathectomy, the activity per LN was even more pronounced.

When immune LN cells from such 6-OHDA-treated mice were tested for secondary ( $2^\circ$ ) in vitro generation of CTL, their response was not affected relative to vehicle controls (Table 6). Different requirements exist for such  $2^\circ$  responses since immune cell populations contain increased frequencies of T helper (Th) cells and CTL precursors (in addition to effector CTL). Therefore, even though effector CTL activity was reduced following sympathectomy, sufficient activation of Th cells and CTL precursors may have occurred to allow a normal  $2^\circ$  response in vitro. Future experiments will be directed toward this interesting observation.

Two weeks of chronic beta blockade in vivo by propranolol also reduced significantly the CTL response in LN following alloimmunization (Table 7). Coupled with the similar effects of acute 6-OHDA, this finding suggests that innervation and beta adrenergic stimulation must be at normal levels for intact CTL responses. Cells from primed LN generated normal  $2^\circ$  in vitro CTL responses, just as with 6-OHDA. Possibly, different cell populations induced during alloimmunization in vivo vary in their content of beta adrenergic receptors or in their sensitivity to sympathetic (noradrenergic or adrenergic) stimulation. In vitro studies with specific agonists are needed to address this point. Differences in suppressor cell activation in vivo may also be involved. On the other hand, these reductions in CTL activity may be due solely to alterations in the cellular composition of the LN as a result of neural manipulation. In either case, the fact that acute 6-OHDA

effects and chronic propranolol effects are similar in magnitude and direction supports the contention that the sympathetic nervous system, via noradrenergic innervation of lymphoid organs, may play a permissive role in the regulation of immune function.

In another experiment (Table 6), we tested the primary *in vitro* MLC proliferative and CTL responses of spleen cells from 6-OHDA treated mice. Since we used pooled cells from several animals in each group, no error terms are shown. (The variability between replicate microplate wells in both assays was negligible.) We found that the proliferative response of non-immune spleen cells was reduced by about 50%, as was the level of CTL activity generated by these cells.

Somewhat similar results were obtained when mice were subjected to propranolol treatment for 4 wk (the only interval examined thus far). As in denervated mice (Fig. 5), spleen cells of beta blocked mice (4 wk, Table 7) showed depressed MLC proliferation, indicating an alteration in IL2 production and/or responsiveness. However, in contrast to the 6-OHDA treatment, which also showed inhibition of CTL generation, the propranolol treatment did not alter the CTL response. Clearly, both types of experiments require replication, and the time course of treatment must be expanded. Since the MLC proliferative response of unprimed cells is largely a reflection of Th cell activity, Th cells may be particularly sensitive to sympathetic neural influence in their unprimed state. Once primed, however, cellular sensitivity may change, or the altered requirements of the 2° CTL response (discussed above) may simply be less sensitive to a partial reduction of Th cell activity.

These results are consistent with our earlier observation that 6-OHDA reduced primary PFC responses in draining LN in response to s.c. immunization. Although numerous possibilities exist to explain these effects, the action of Th cells is common to both antibody and cell-mediated responses. Since Th cells exert their effects in large part through the release of IL2 and other growth- and differentiation-promoting lymphokines, IL2 production may be a target for neural modulation, especially in the non-immune state. Therefore, in addition to the necessary functional studies, anatomical studies to determine the localization of lymphocytes bearing markers of Th cells and IL2 receptors in relation to noradrenergic nerve fibers will be important in this context.

#### e. Effects of adrenergic agents *in vitro*.

Sympathetic neural influences in lymphoid tissues are most likely mediated by the paracrine effects of released NE (and other co-localized amine and peptide neurotransmitters). Thus an understanding of the effects of manipulation of this system *in vivo* would be served by concurrent study of direct effects of such transmitters on immune responses *in vitro*. We have begun to develop evidence that a prevailing notion, that adrenergic stimulation is "immunosuppressive" (which came from experiments using high doses of drugs and the measurement only of mitogen responses), may be inaccurate or, at least, oversimplified. We have directed our efforts to the *in vitro* CTL response, which involves antigen presentation, activation and action of Th cells, and the proliferation and differentiation of CTL precursors (CTLp), as a target system in which the role of noradrenergic and adrenergic interactions with the immune system can be studied. This decision was also prompted by our observations of the *in vivo* effects of sympathetic manipulation, and our cognizance of the important role CTL play in anti-viral immunity.

Generation of CTL activity from mixtures of normal C3H spleen and LN cells in bulk flask culture, stimulated by irradiated allogeneic BALB/c spleen, was studied. In a large series of experiments, a number of non-selective (NE and epinephrine (EPI)) as well as beta- and alpha- selective adrenergic compounds were tested for their ability to modulate the CTL response. As depicted in Fig 6, NE and EPI in the nanomolar to micromolar concentration range potentiated the CTL response, sometimes by as much as 500%. We have not yet been able to distinguish satisfactorily the receptor specificity of this response.

While mixed agonists had potent activity, a beta<sub>1</sub>-selective agonist, dobutamine had no effect, consistent with the presumed beta<sub>2</sub> nature of the lymphoid cell adrenergic receptor. As predicted, stimulation with a beta<sub>2</sub>-selective agent, terbutaline, had enhancing activity, while alpha stimulation with methoxamine also appeared to potentiate the response.

Several receptor antagonists were tested for their ability to block the agonist-induced potentiation of the CTL response (Fig. 7). The difficulty with this approach is the general membrane-inhibiting (local anesthetic) activity of some of these compounds at desired concentrations. The beta blocker propranolol or the alpha blocker phentolamine each gave a partial inhibition of the EPI- or NE-induced potentiation, at equimolar concentrations. Given the predominant beta selectivity of EPI, the lack of additivity of inhibition by propranolol and phentolamine may indicate that part of the antagonist activity (or all of the phentolamine activity) is non-specific. There is additivity of inhibition of the NE response, which should act via both beta- and alpha- receptors. This would lead to a tentative conclusion that stimulation of either beta<sub>2</sub> and/or alpha receptors, presumably on lymphocytes, promotes some activity leading to enhanced generation of CTL. These findings are in agreement with the in vivo inhibitory effects of sympathetic denervation and beta receptor blockade on these functions. Further in vitro studies with a beta blocker which has less non-specific local anesthetic effects than propranolol, allowing the use of higher concentrations, would be important. Timolol shows promise for this purpose, since concentrations as high as 1 mM did not inhibit CTL generation.

As a first attempt to understand the mechanisms of the potentiated CTL responses, we analyzed the yields of viable cells from the cultures in which CTL were generated. In general, the cell yields were not significantly different from controls at concentrations of agonists that induced significant increases in CTL activity. Propranolol tended to inhibit proliferation at higher concentrations, while phentolamine did not have this activity. This lack of a clearcut increase in cell yields would suggest that the enhanced CTL activity is related to greater lytic capacity per cell generated under these conditions.

An experiment testing the effects of adrenergic agents on the MLC proliferative response of normal and alloimmune LN cells (in microculture) was performed next, and the results appear in Table 8. At a concentration of 1  $\mu$ M, all 3 agonists significantly increased thymidine incorporation by immune and normal LN cells. Therefore, part of the total increase in CTL activity generated can be attributed to a greater proliferative response, presumably reflecting greater IL2 production/responsiveness, as well as more CTL. More detailed studies, including limiting dilution analysis of precursors of IL2 producing cells and of CTL are required to dissect these effects, and determine precisely which cell populations, at what stage of the response, are subject to influences via different adrenergic receptors. Varying the timing of addition, or removal, of agonists and antagonists in the cultures should also help determine the target(s) of the potentiating effects.

Based on our results, and the work of Sanders and Munson examining in vitro antibody responses, it is clear that a much more extensive analysis is necessary to begin to understand interactions of neurotransmitters with cells of the immune system, even in an in vitro response. Broader dose ranges of agents must be tested due to "peculiar" dose-response curves. For example, clonidine, a presumptive alpha<sub>2</sub>-selective agonist, has been reported to suppress antibody responses in vitro. We have observed a tri-phasic dose-response curve with CTL responses in 2 separate experiments, examining a 6 log concentration range of clonidine from 0.1 mM to 0.1 nM (not shown). This could be interpreted as presence of alpha<sub>2</sub> receptors on lymphocytes, which is supported by direct binding studies to membranes of guinea pig spleen. However, recent description of a "clonidine receptor" suggests that the situation may be more complicated.

#### f. Effects of stressful stimuli on immunological function

Several experiments were performed using cold water (10°C) as an acute stressor. C3H mice were forced to swim in cold water for 3 intervals of 5 min, separated by 2 min. rest periods per day. Mice showed decreased NK cell activity 24 hr after 1-3 days of such stress. T and B lymphocyte responses to mitogens of cells from the same spleens were unaffected. Similarly, in vivo generation of CTL following alloimmunization was not affected by as many as 4 daily sessions of cold swim, whether imposed prior to immunization, between immunization and testing, or both.

Based on the lessons learned from these paradigms thus far, we will concentrate most intently on NK cell function in the spleens of mice subjected to cold stress. Subsequent studies of natural resistance to HSV1 will be designed in accordance with the results obtained from study of NK. Examination of the effects of cold stress on specific immune responses, including contact sensitivity and anti-HSV1 reactions, will be performed later.

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Table 1.

## CONTACT SENSITIVITY RESPONSES TO REACTIVE HAPTENS

Mice	Immunizing Hapten <sup>^</sup>	Challenge Hapten	Ear Swelling Response <sup>#</sup> at:			
			n	24 hr	n	48 hr
BALB/c	---	TNP	4	17 $\pm$ 3		
		DNP	3	10 $\pm$ 0		
		OXA	4	13 $\pm$ 8		
	TNP	TNP	17	61 $\pm$ 11*	9	42 $\pm$ 6*
		DNP	8	8 $\pm$ 6	4	0 $\pm$ 0
		OXA	8	13 $\pm$ 6	3	20 $\pm$ 12
	DNP	TNP	4	15 $\pm$ 3		
		DNP	4	35 $\pm$ 7*		
		OXA	3	20 $\pm$ 4		
	OXA	TNP	4	15 $\pm$ 7		
		DNP	3	10 $\pm$ 0		
		OXA	5	57 $\pm$ 8*		
	TNP	TNP	5	92 $\pm$ 15*	5	53 $\pm$ 9*
		DNP	5	12 $\pm$ 6	5	27 $\pm$ 9

<sup>^</sup> Haptens used were trinitrophenyl (TNP) administered as 7% trinitrochlorobenzene (picryl chloride), dinitrophenyl (DNP) administered as 0.5% dinitrofluorobenzene, and oxazolone (OXA) administered as 3% 4-ethoxymethylene-2-phenyloxazolone.

<sup>#</sup> Response is measured as mean ear swelling (inches  $\times 10^{-4}$ )  $\pm$  S.E.M. at 24 or 48 hrs after challenge.

\* Statistically significant responses ( $p < 0.05$ , Student's  $t$  test, compared to appropriate controls).

Table 2.

## EFFECT OF ADULT SYMPATHECTOMY ON DELAYED HYPERSENSITIVITY RESPONSES

Mice	6-OHDA Dose (mg/kg)	Time (day)*	n	Response (Percent of Control)#	
				24 hr	48 hr
BALB/c	Control		16	100 $\pm$ 4	100 $\pm$ 9
	100	-3,-1	6	82 $\pm$ 11	90 $\pm$ 15
	150	-3,-1	11	72 $\pm$ 6 <sup>b</sup>	63 $\pm$ 7 <sup>c</sup>
	300	-3,-1	4	74 $\pm$ 6 <sup>a</sup>	66 $\pm$ 23 <sup>a</sup>
C3H	Control		12	100 $\pm$ 5	100 $\pm$ 5
	150	-1	5	100 $\pm$ 11	58 $\pm$ 12 <sup>b</sup>
	150	-3,-1	4	54 $\pm$ 12 <sup>c</sup>	73 $\pm$ 24
	150	+4	5	71 $\pm$ 8 <sup>b</sup>	96 $\pm$ 11
	150	+2,+4	5	81 $\pm$ 5 <sup>a</sup>	95 $\pm$ 16

\* Time of drug injection relative to day of sensitization (day 0) with trinitrochlorobenzene (TNCB). Ear thickness was measured and ears were challenged with TNCB on day 5. The response was measured 24 and 48 hrs later.

# Mean ( $\pm$  S.E.M.) ear swelling (inches  $\times 10^{-4}$ ) expressed as percent of the mean of the appropriate vehicle control group.

Values significantly different by Student's t test from vehicle controls are indicated by superscripts.

- a:  $p < 0.05$
- b:  $p < 0.01$
- c:  $p < 0.001$

Table 3.

## DELAYED HYPERSENSITIVITY RESPONSES TO ALLOANTIGENS

STRAIN Host - Donor	SENSITIZATION		CHALLENGE		N	DH RESPONSE <sup>+</sup>
	DOSE (cells x 10 <sup>-6</sup> )	SITE	[Strain] (cells x 10 <sup>-6</sup> )			
C3H anti- B6D2F <sub>1</sub>	10	Footpads	[B6D2F <sub>1</sub> ] 20	4	248	± 19
	20	Footpads, Base of tail	20	7	272	± 21
	20	Footpads, Base of tail, Inguinal	10	4	263	± 14
BALB anti- C3H	20	Footpads, Base of tail	[C3H] 20	3	180	± 42
			10	4	195	± 43
			5	3	120	± 55
			2.5	3	30	± 10 <sup>n</sup>
			1.2	3	37	± 7 <sup>n</sup>
			saline	4	27	± 3
			[C57BL/6] 10	4	25	± 5 <sup>n</sup>
			[AKR] 10	4	98	± 18

<sup>+</sup> 24 hr footpad swelling (inches x 10<sup>-4</sup>)

<sup>n</sup> Not significantly different from saline control

**Table 4.**  
**LOCAL TRANSFER OF DH TO ALLOANTIGENS**

<u>Immune LN Cells Transferred <sup>^</sup></u>	<u>Number (x 10<sup>-6</sup>)</u>	<u>Antigen Challenge*</u>	<u>Recipients (strain)</u> <u>N</u>	<u>Footpad Swelling<sup>#</sup></u>
			(BALB/c)	
None	—	C57BL	3	37 ± 3
BALB non-immune	10.	C57BL	3	77 ± 9
BALB anti-C57BL	0.5	C57BL	4	50 ± 9 <sup>n</sup>
	1.	"	4	75 ± 12
	2.	"	4	113 ± 5
	5.	"	4	133 ± 13
	10.	"	4	133 ± 16
	5.	none	3	27 ± 13 <sup>n</sup>
BALB anti-C3H	10.	C3H	6	247 ± 24
			(C3H)	
C3H anti-DBA/2	7.5	C3H	3	53 ± 20 <sup>n</sup>
	"	DBA/2	4	238 ± 9
	"	BALB/c	3	173 ± 9
	"	C57BL	3	122 ± 9

<sup>^</sup> Mice were immunized S.C. 4-6 days earlier with 30 x 10<sup>6</sup> allogeneic spleen cells distributed in both footpads, the base of the tail, and the inguinal regions.

\* Antigen challenge consisted of 10 x 10<sup>6</sup> spleen cells of the indicated mouse strain, mixed with the indicated number of adoptively transferred LN cells, in a volume of 50 ul BSS, injected S.C. into the right footpad. The left footpad received an equal volume of BSS. (Earlier experiments showed no difference between BSS and syngeneic spleen cells.)

<sup>#</sup> Footpad swelling, measured in inches x 10<sup>-4</sup>, was determined 24 hrs later and expressed as the difference between the thickness of the right (experimental) and left (control) feet.

<sup>n</sup> Not significantly different from negative control.

**Table 5.**  
**EFFECT OF BETA RECEPTOR BLOCKADE ON ANTIBODY RESPONSES**  
**TO SRBC IN VIVO**

<u>Propranolol<sup>+</sup></u> <u>Treatment</u>	<u>N</u>	<u>PFC / 106<sup>#</sup></u>	<u>PFC / Organ</u> <u>(x 10<sup>-3</sup>)</u>
<u>SPLEEN (i.p. imm.)</u>			
Control	8	948 ± 84	133.3 ± 18.0
Acute (d. -1,+1) <sup>†</sup>	4	1083 ± 220	133.2 ± 26.9
Chronic (3 wks)	5	1935 ± 86*	219.6 ± 10.5*
Chronic (4 wks)	4	941 ± 231	128.0 ± 40.5
<u>LYMPH NODE (s.c. imm.)</u>			
Control	6	386 ± 74	3.6 ± 0.7
Acute	4	394 ± 135	1.6 ± 0.6
Chronic (3 wks)	4	253 ± 78	0.8 ± 0.2*
Chronic (4 wks)	4	738 ± 62*	5.7 ± 1.1

+ Chronic propranolol: 2 mg pellets implanted s.c., resulting in daily dosage of approximately 3 mg/kg

† Acute propranolol: 3 mg/kg

# PFC values appear as mean ± s.e.m.

\* Significantly different from control (p < 0.05, Student's t test)

There is a significant effect of drug treatment (one-way ANOVA) on both spleen and LN PFC responses.

Table 6.

SYMPATHECTOMY INFLUENCES ALLOREACTIVE T CELL RESPONSES IN VITRO

<u>C3H Donor</u>	<u>6-OHDA<sup>#</sup></u>	<u>Cells</u>	<u>Cytotoxic<sup>†</sup> Activity</u>	<u>Proliferative<sup>††</sup> Response</u>
Non-Immune	—	SPL	8.4	12,400
	++	SPL	4.5*	6,200*
Alloimmune	—	LN	40.0	
	++	LN	34.5	

<sup>#</sup> 2 injections of 100 mg/kg on d. 1 and 3 before immunization (d. 6 and 9 before culture).

<sup>†</sup> Lytic units<sub>30</sub> per 10<sup>6</sup> cultured responder cells.

<sup>††</sup> Net CPM of <sup>3</sup>H-thymidine incorporation by 2 x 10<sup>5</sup> responders

\* Significantly different from vehicle control.



Table 7

## EFFECT OF CHRONIC PROPRANOLOL ON ALLOREACTIVE T CELL RESPONSES

WEEKS OF DRUG <sup>#</sup>	ASSAY (Units)	-GRP	N	RESPONSE (Mean $\pm$ sem)	SIGNIF. <sup>+</sup>
2	<u>LYMPH NODE</u>				
	CTL IN VIVO	Drug	4	5.5 $\pm$ 0.5	p<0.05
	(LU / 10 <sup>6</sup> cells)*	Veh	3	7.1 $\pm$ 0.5	
	2 <sup>o</sup> CTL IN VITRO	Drug	4	41.5 $\pm$ 6.1	N.S.
	(LU/10 <sup>6</sup> cult. cells)	Veh	3	43.8 $\pm$ 9.7	
4	<u>SPLEEN</u>				
	MLC PROLIFERATION	Drug	5	130 $\pm$ 7	p<0.01
	(net cpm $\times$ 10 <sup>-3</sup> )	Veh	3	79 $\pm$ 6	
	1 <sup>o</sup> CTL IN VITRO	Drug	5	13.6 $\pm$ 0.8	N.S.
	(LU / 10 <sup>6</sup> cells)	Veh	3	10.9 $\pm$ 1.3	

# Chronic propranolol: 2 mg pellets implanted SC, resulting in daily dosage of ~ 3 mg/kg. Vehicle controls were implanted with placebo pellets.

\* Significant differences from vehicle control, Student's t test

• LU: Lytic units. LU<sub>30</sub> for LN CTL induced in vivo; LU<sub>50</sub> for in vitro generated 2<sup>o</sup> LN CTL and 1<sup>o</sup> spleen CTL.

Table 8

**EFFECT OF ADRENERGIC AGENTS ON T CELL  
PROLIFERATIVE RESPONSE TO ALLOANTIGENS IN MLC**

<u>COMPOUND</u>	<u>CONC.</u> <u>(<math>\mu</math>M)</u>	PERCENT INCREASE IN RESPONSE BY:*	
		Normal LN <u>cells</u> <sup>a</sup>	Immune LN <u>cells</u> <sup>b</sup>
<u>A. Agonists</u>			
NOREPINEPHRINE	100	(8) <sup>c</sup>	25
	10	29	(-2) <sup>d</sup>
	1	33	10
EPINEPHRINE	100	(-10)	(4)
	10	(19)	11
	1	29	40
ISOPROTERENOL	100	(-4)	(-9)
	10	42	34
	1	50	11
<u>B. Antagonists</u>			
PROPRANOLOL	100	-25	-99
	10	29	(8)
	1	(20)	16
PHENTOLAMINE	100	(-19)	-99
	10	(2)	14
	1	(-24)	49

\* Response is incorporation of <sup>3</sup>H-thymidine by C3H LN cells during final 16 hr of 72 hr MLC (BALB/c stimulators). Percent increase (or decrease) is relative to control cultured with no drug.

<sup>a</sup> 2 x 10<sup>5</sup> non-immune LN responder cells

<sup>b</sup> 1 x 10<sup>5</sup> alloimmune LN responder cells

<sup>c</sup> Numbers enclosed in parentheses are NOT significantly different from control values (i.e. they are within the 2 Std. Dev. range of controls)

<sup>d</sup> Negative numbers indicated a decrease (rather than an increase) from control values

The 2 Std. Dev. ranges of the control groups were:

Normal LN: -25% to +25% of control

Immune LN: -9% to +9% of control

FIGURE 1

IL2 PRODUCTION IN BALB/C ANTI-TNP RESPONSE

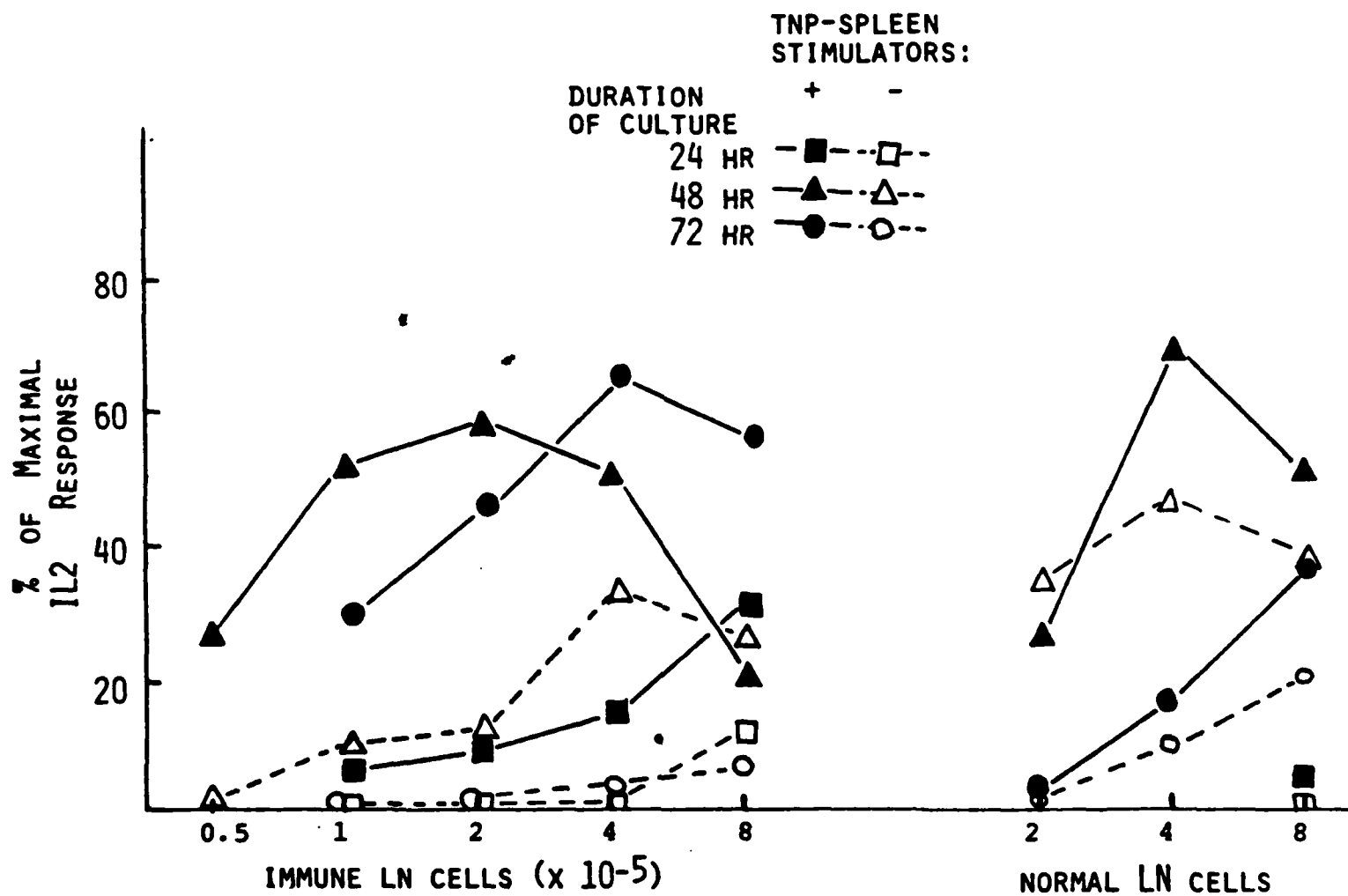


FIGURE 2.

SYMPATHECTOMY AUGMENTS BOTH BASELINE AND  
POLY I:C-STIMULATED NK CELL ACTIVITY IN SPLEEN

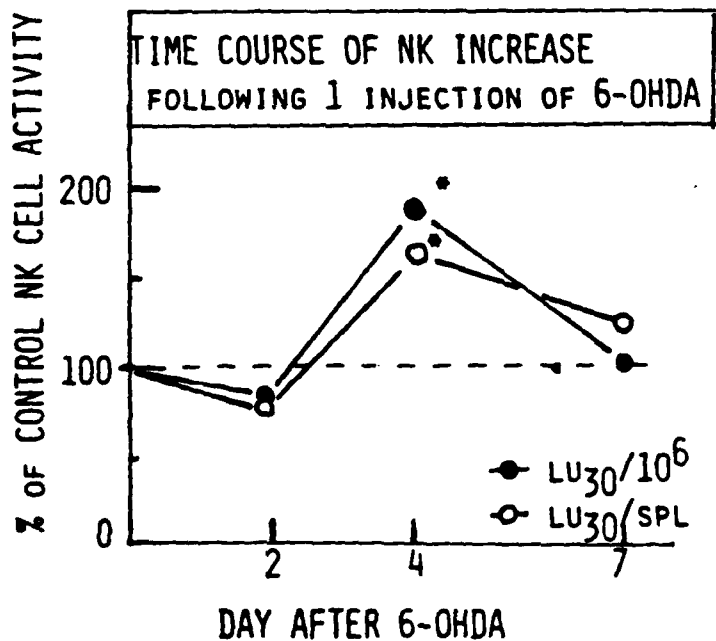
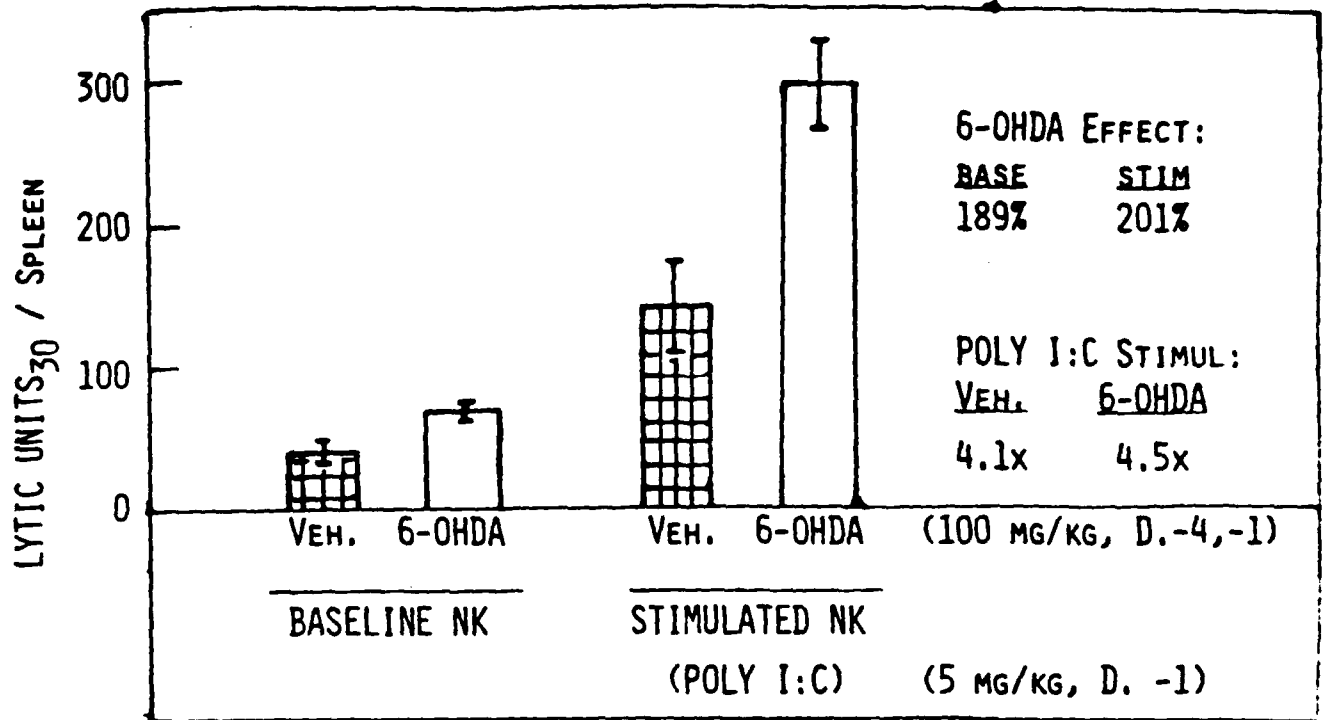
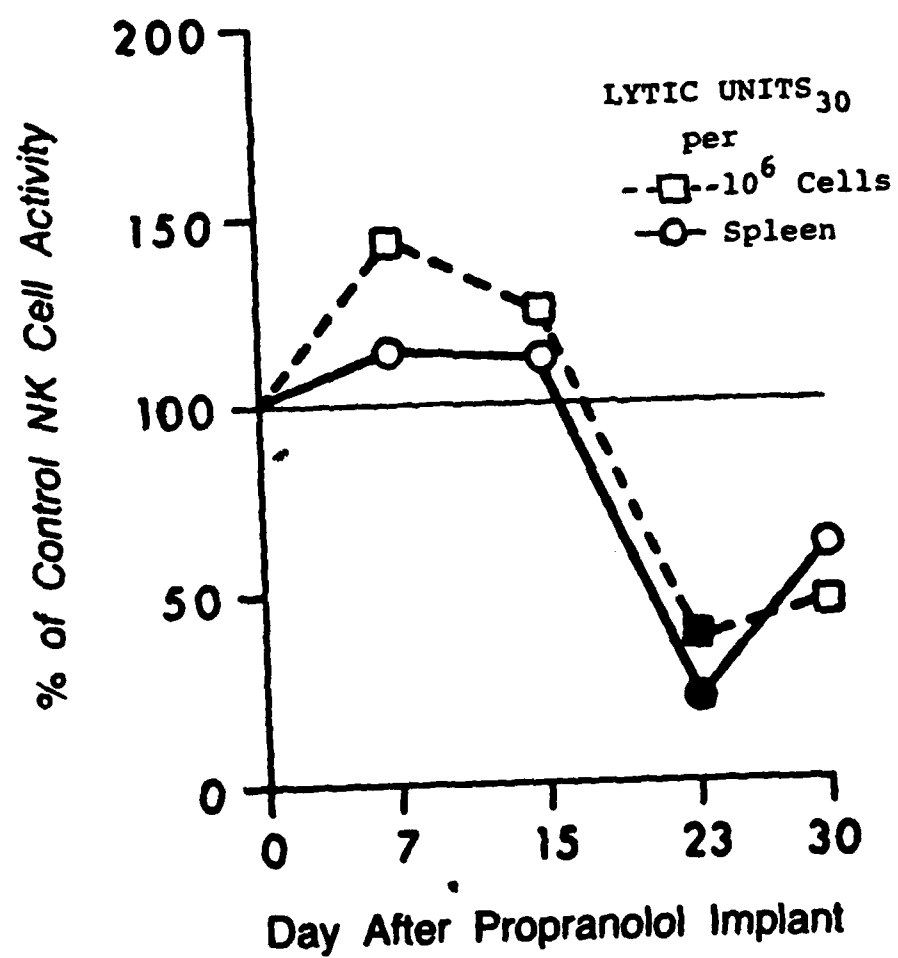
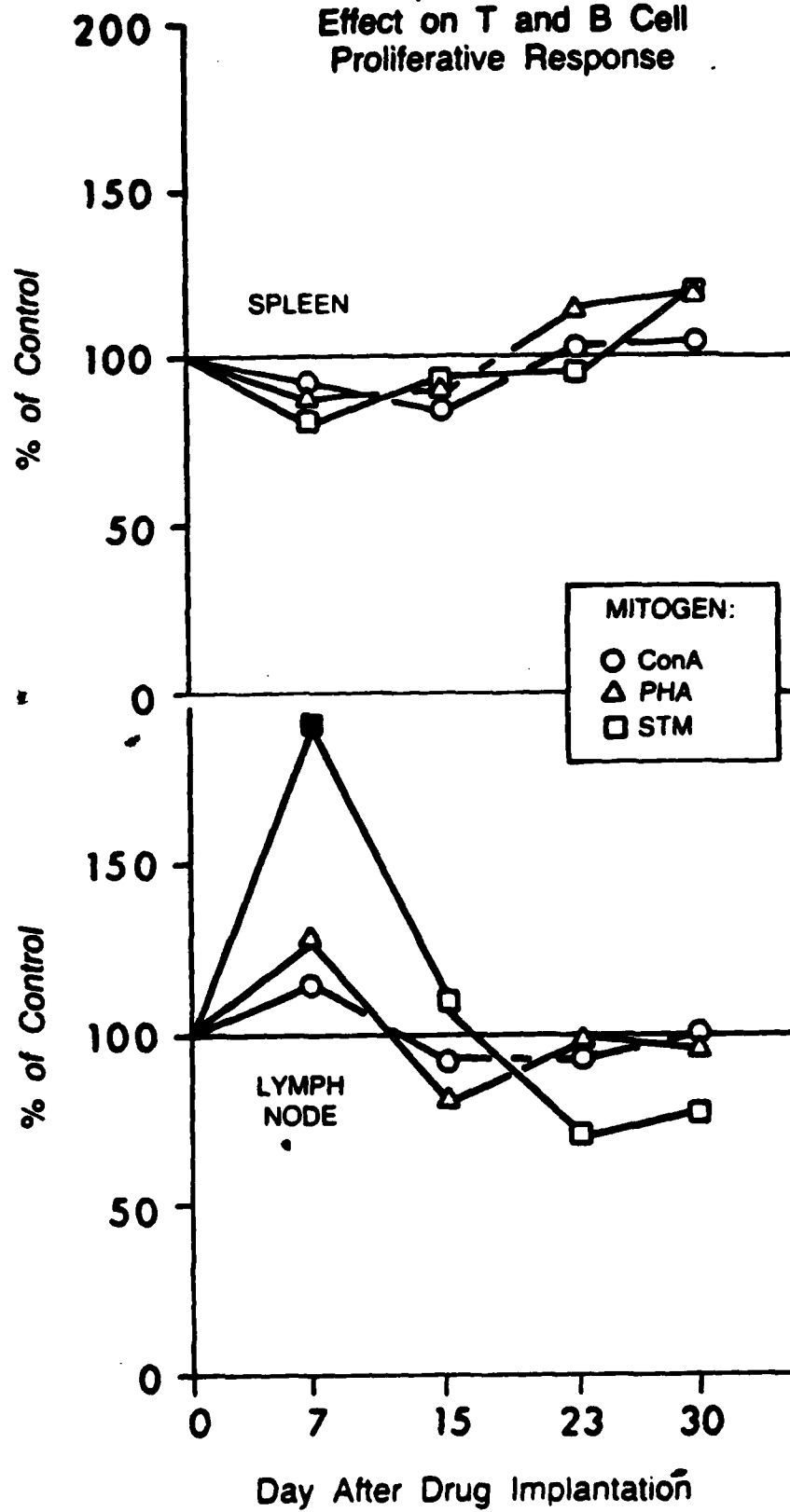


FIGURE 3.



**FIGURE 4**

**Chronic Propranolol Administration:  
Effect on T and B Cell  
Proliferative Response**



**FIGURE 5**

**SYMPATHECTOMY SUPPRESSES IN VIVO  
GENERATION OF CTL**

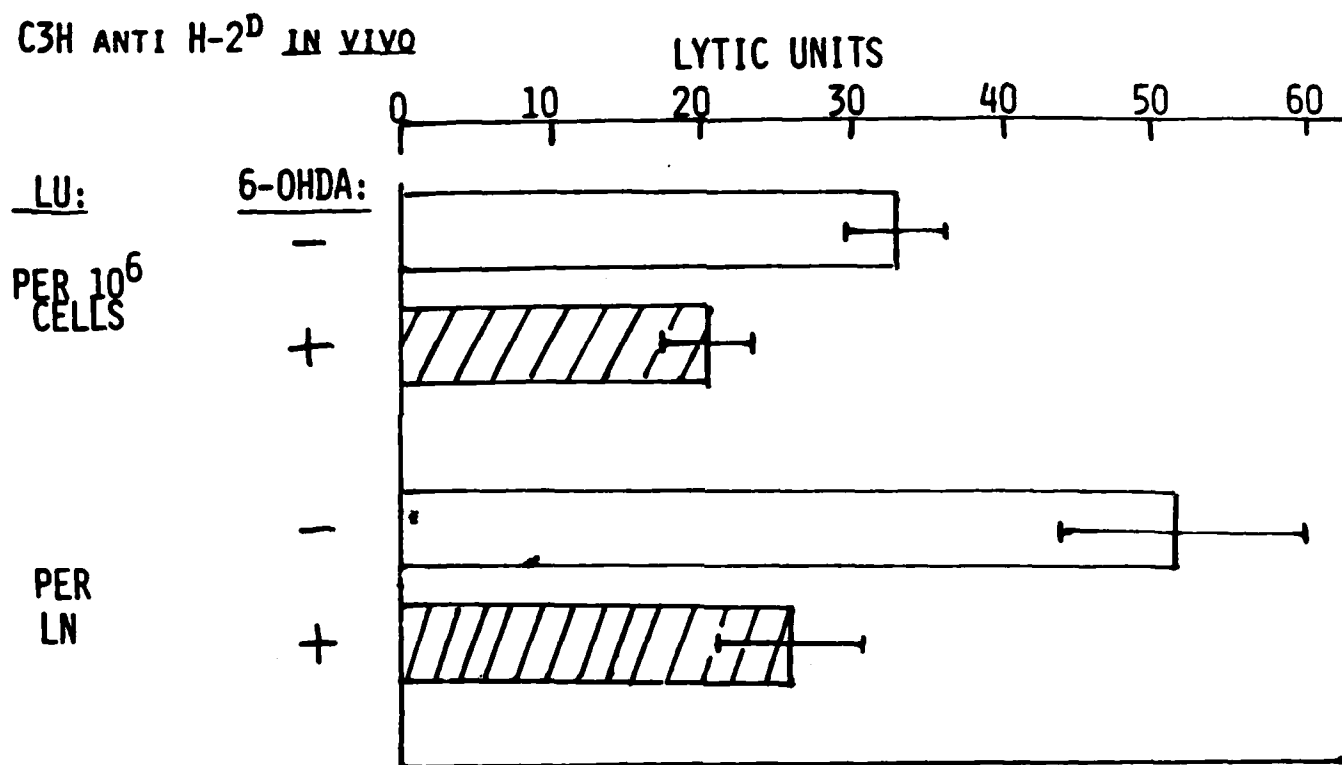
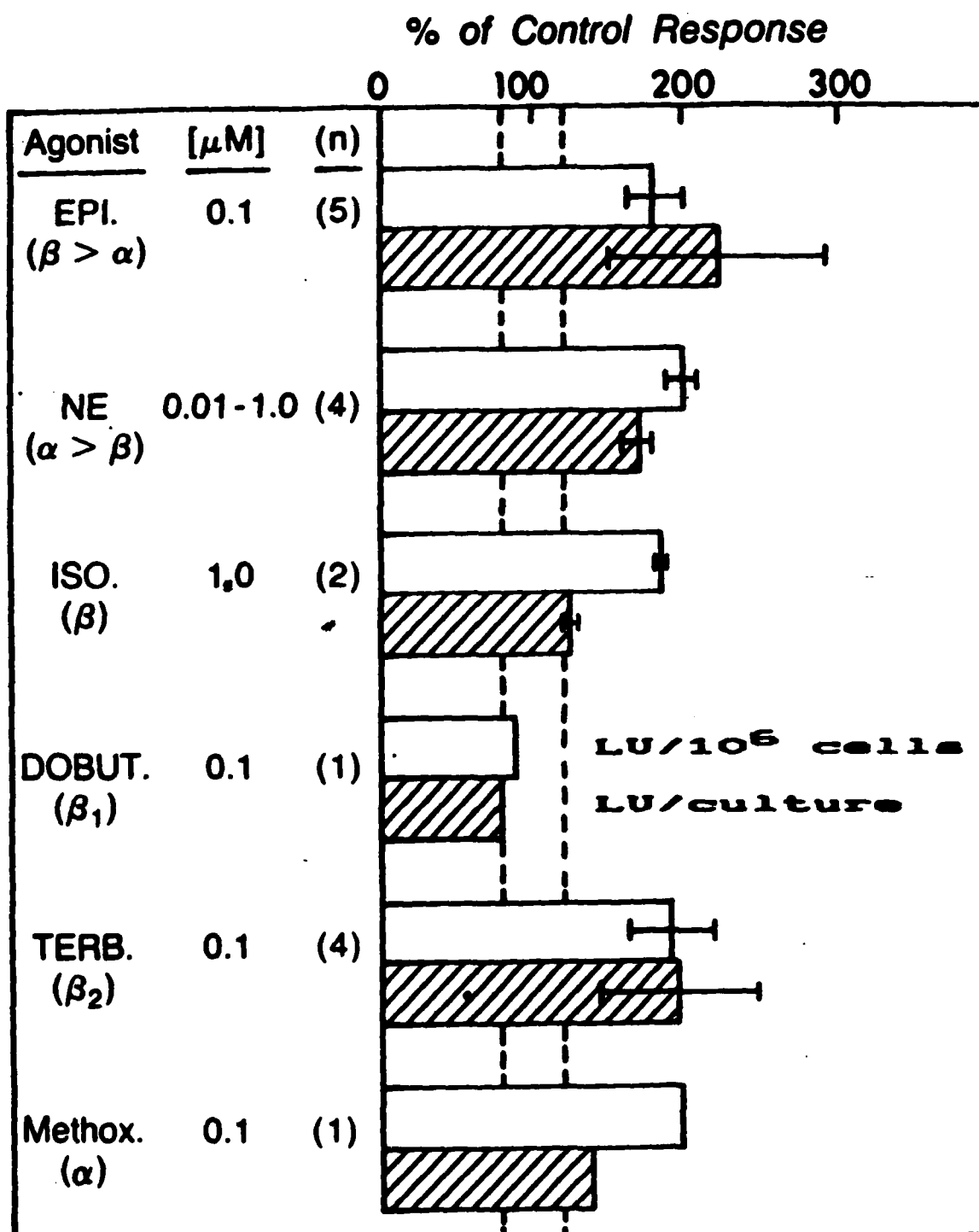


FIGURE 6.

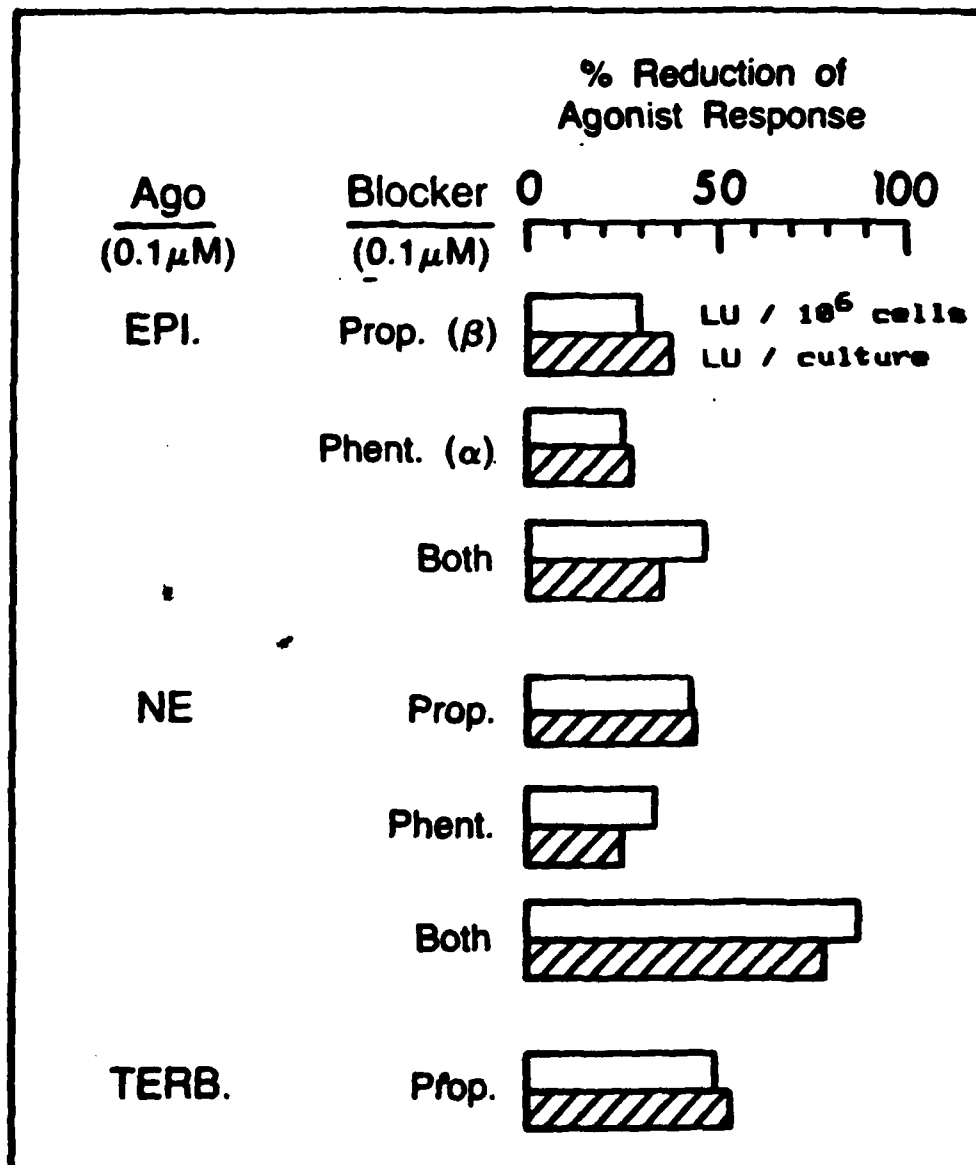
**Potentiating Effects of Adrenergic  
Drugs on Primary Generation of  
Cytotoxic T Cells *In Vitro***





**FIGURE 7.**

**Adrenergic Blockers Inhibit Potentiated  
Cytotoxic T Cell Responses**



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